

**Sal k 5, a member of the ubiquitous Ole e 1-like protein family, is a relevant allergen of Russian thistle (*Salsola kali*) pollen**

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**Short title** Sal k 5, an allergenic Ole e 1-like member from *Salsola kali*

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## Summary

**Background:** *Salsola kali* is an Amaranthaceae weed with important repercussions in pollinosis of temperate areas. Ole e 1-like members are relevant allergens in pollen from different species. We aimed to characterize and produce as recombinant allergen *S. kali* Ole e 1-like protein.

**Methods:** The natural allergen was purified at homogeneity after three chromatographic steps. Specific cDNA was sequenced and expressed in *Pichia pastoris* yeast. Structural relationships of natural and recombinant forms were carried out by 2D-electrophoresis and spectroscopic analyses. Its immunological relevance was analyzed by ELISA and immunoblotting using an IgG antiserum and monoclonal antibodies specific to Ole e 1, as well as sera from fifty-seven allergic patients recruited from two Spanish regions where this pollinosis is frequent.

**Results:** The purified allergen, Sal k 5, is an acidic glycoprotein of 151 amino acid residues and 17628 Da of molecular mass. Its amino acid sequence exhibits 68% and 32% of identity with the allergens of this family Che a 1 and Ole e 1, respectively. The recombinant protein was correctly processed and its structural and immunologic equivalence to the natural form was proved. A sensitization frequency up to 30% was observed in pollinic patients from the center and coastal east of Spain.

**Conclusions:** Sal k 5 is a member of the Ole e 1-like protein family, which can be considered an important allergen from *S. kali*. Its inclusion in diagnosis protocols would allow accurately defining patients allergic to this pollen.

## Introduction

Soil desertification of extensive zones from the Mediterranean area is enhancing the incidence of certain widespread weeds of temperate areas and salt soils, such as Chenopodiaceae species that have been recently included within Amaranthaceae family [1]. Sensitization to pollen from these species has constituted a severe problem in semi-desertic countries such as Iran, Kuwait and Saudi Arabia [2,3]. The resistance to drought of these plants, the use of several species in hedges, ornamental decoration and irrigated crops, as well as its presence as contaminant weeds in culture areas such as those of olive trees, have made these plants important pollinosis inducers in the Mediterranean area [4-6]. Although Russian thistle (*Salsola kali*) and Lamb's quarter (*Chenopodium album*) are the most representative pollens of the family in terms of allergenic incidence in Spain [6-9], other species of the Amaranthaceae family as *Salsola pestifer*, *Amaranthus retroflexus* or *Salsola oppositifolia* have been reported as allergenic species [10,11].

Although these weeds have been currently considered as minor sources of allergens because they count for a reduced number of pollen grains, up to 30 to 40% of the sensitized individuals exhibiting symptoms at the end of summer in several regions of Spain are allergic to these aggressive pollens. Nowadays, in Spain, *Salsola* pollen is the third more relevant cause of pollinosis after olive and grasses [6], either in regions where allergy to this pollen is one of the major sensitization causes, as for instance Zaragoza and Murcia, or in those regions such as central Spain where the wild growth of this weed is concomitant to olive tree cultivars [4,12].

Three allergens have been identified and characterized in *C. album* pollen: Che a 1, a member of the Ole e 1-like protein family, the profilin Che a 2 and the polcalcin Che a 3 [7]. Regarding *S. kali* allergens, Sal k 1, a pectin methylesterase, has been described so far as major allergen and specific marker of *S. kali* pollen [9], and several minor allergens reported: Sal k 3, a methionine synthase [13] and the profilin Sal k 4 [14,15]. In addition, sera of patients allergic to *S. kali* contain IgEs able to bind to other proteins in immunoblotting with high frequency such as a 19 or 25 kDa bands that are recognized by a significant number of patients from several Spanish populations. Considering the existence in pollens of an ubiquitous protein family with molecular masses of around 19 kDa, whose representative member is Ole e 1, the major allergen from *Olea europaea* pollen [16], and having into account that an Ole e 1-like allergen -Che a 1- has been already identified in *C. album* [17], our aim was focused on identifying and characterizing an homologous member in *S. kali* pollen with significant allergenic character.

This broad group of allergens has been ubiquitously detected in different plant species, being exclusively expressed in pollens and exceptionally in leaves such as that of *Sambucus nigra* [18]. These cysteine-rich proteins seem to play an essential biological role during hydration/germination and pollinic tube growth [19]. Some of these molecules Ole e 1, Syr v 1, Lig v 1 and Fra e 1 are major allergens in Oleaceae pollens [9,20,21]. Other allergens of the family belonging to taxonomically unrelated species such as Lol p 11 from Gramineae and Pla l 1 from Plantaginaceae have a lower allergenic incidence in the Mediterranean area [22,23]. Although the allergenic character is associated to this protein family, a non allergenic member has been recently identified in birch [24]. The fact that this family contains important aeroallergens with significant repercussions in allergy manifestations of many populations makes its identification essential for diagnostic purposes.

In this work, we report the isolation and characterization of an allergenic Ole e 1-like protein from *S. kali* pollen, as well as the analysis of its relevance among patients allergic to this pollen, whose increasing allergenic relevance in South Europe make necessary its inclusion in the diagnosis trials. A recombinant form has been produced in *P. pastoris* and validated using the natural form, and thus, the availability of this protein would allow using it as a tool for diagnosis of allergic patients to this species widely distributed in semi desert areas.

## Materials and methods

### *Pollen extracts, S. kali sensitized patients' sera and antibodies*

*S. kali*, *C. album*, and *O. europaea* protein extracts were prepared using pollen provided by ALK-Abelló (Madrid, Spain) as previously described [9]. Recombinant Che a 1 and Ole e 1 allergens were purified according to [17,25]. Sal k 1 was purified according to [9] and Sal k 4 was obtained as reported [15].

Fifty-seven patients sensitized to *S. kali* were recruited from Murcia, a dried coastal region, and Zaragoza, a province from the interior of Spain, fulfilling the following criteria: 1) seasonal rhinitis and/or bronchial asthma from May to September, 2) a positive skin prick test to *S. kali* pollen extract and specific IgE values >0.7 kU/l measured by CAP (Pharmacia Biotech, Piscataway, USA), and 3) no previous *S. kali* immunotherapy (table S1). One non-atopic serum was used as negative control. All patients provided informed consent. The Ethical Committee of the Complutense University (Madrid, Spain) approved the protocols used for experimental work related to the use of human sera in the study.

Rabbit polyclonal antiserum (pAb) against the *O. europaea* allergen nOle e 1 was used [25]. The use of animals for experimental procedures was supervised for the animal facility of the Fundación Jiménez Díaz (Madrid, Spain). Monoclonal antibodies (mAbs) OL3 and OL7 obtained against nOle e 1 were used in the analysis of the structural integrity of this protein [26]. Horseradish peroxidase-labelled goat polyclonal antibody against rabbit IgG was obtained from Bio-Rad (Richmond, California). Mouse monoclonal antibody against human IgE was kindly donated by ALK-Abelló. Horseradish peroxidase-labelled goat polyclonal antibody against mouse IgG was purchased from Pierce Chemical Co. (Rockford, Ill, USA).

### *Isolation of the allergen from S. kali pollen*

Three chromatographic steps, -gel permeation onto a Sephacryl S-100 column in 0.2 M ammonium bicarbonate, pH 8.0, hydrophobic chromatography on a phenyl-sepharose column and eluted with a reverse hydrophobicity gradient (5 to 0 M) of NaCl, and a reverse-phase HPLC on a Nucleosil C<sub>18</sub> column with an acetonitrile gradient (0-60%) in 0.1% trifluoroacetic acid - were used for the purification of the allergen from *S. kali* pollen.

### *Cloning of Sal k 5*

Total RNA from *S. kali* pollen was extracted as described [9]. cDNA was synthesized using the SMART RACE cDNA amplification kit (BD Biosciences-Clontech, Madrid, Spain). Specific cDNA was amplified by two rounds of PCR using four specific primers. In the first step two internal primers: 5'-GTNTAYTGYGAYACNTGYCG-3' (VYCDTCR) and 5'-RTCRTCYTCRTGRTCNCRCRTC-3' (DGDHEDD), which were designed using the peptides sequences in parentheses obtained from mass spectrometry analysis, were used to obtain the nucleotide sequence encoding a polypeptide internal fragment of 60 amino acid residues. Afterwards, a second round was performed with the oligonucleotides: antisense 5'-ACCCTCCATCATGGTGCTAAC-3' (VSTMMEGA) and sense 5'-ATGTACACCATCAAAGTCGACGG-3' (MYTIKVDG) primers and the UPM unspecific primer included in the kit to get the whole nucleotide sequence of the protein. Finally, a PCR round with a sense 5'-ccgctcgagaaaagaAARGGNAAYGGNGGNCAAYCT-3' (KGNGGHNL) and an antisense 5'-ctgcccgcgcTCARTTYTGNGTNACRCTNCCNGG-3' (PGSVTQN) primers corresponding to the N and C-terminal ends of the mature protein. The sense and the antisense primers contained a *Xho*I and a *Not*I restriction site respectively (both underlined). The purified DNA was directly cloned into the pCR2.1 plasmid included in the TOPO TA-cloning kit (Invitrogen, Groningen, The Netherlands), used to transform TOP10 *E. coli* cells and sequenced [27]. The DNA fragment corresponding to Sal k 5 was digested with *Xho*I/*Not*I restriction enzymes and subcloned into the same sites of the pPICZ $\alpha$ A plasmid using DH5 $\alpha$  *E. coli* cells as host.

#### *Expression and isolation of recombinant Sal k 5*

pPICZ $\alpha$ A/Salk5 was linearized with *Sac*I restriction enzyme, and used to electroporate KM71 strain of *P. pastoris* cells. Transformed (His<sup>+</sup> Mut<sup>s</sup>) cells were incubated on YPDS/zeocin at 30 °C for 72h in buffered glycerol complex medium until colonies appeared. The recombinant protein was detected by SDS-PAGE in the extracellular culture medium and after dialysis it was purified as described previously for the natural protein from *S. kali* pollen extract.

#### *Analytical methods*

SDS-PAGE was performed by the method of Laemmli in 15% polyacrylamide gels in the absence or presence of 2-mercaptoethanol (2-ME). Gels were stained with Coomassie

Blue R-250 (Sigma-Aldrich, Saint Louis, USA) or transferred to nitrocellulose membranes. Molecular mass determinations were done with protein markers MW-SDS-70L (Sigma-Aldrich). N-terminal Edman degradation of the protein was performed using an ABI Procise 494 protein sequencer (Life Technologies, Carlsbad, USA).

The concentration of the purified proteins was calculated by measuring the absorbance at 280 nm in a DU-7 spectrometer (Beckman, Barcelona, Spain) using the extinction coefficient ( $E^{0.1\%}$ ) of 0.474 according to ProtParam [28].

#### *Mass spectrometry analysis*

Mass spectrometry (MS) analysis was carried out on a Bruker-Reflex IV Matrix-assisted laser-desorption ionization time-of-flight mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany). Peptide mapping fingerprinting and MS/MS sequencing analyses were carried out using the 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems, Carlsbad, California, USA) according to previously published methods [9].

#### *Spectroscopic characterization*

Circular dichroism (CD) spectra were obtained in the far-UV (190-240 nm) on a Jasco J-715 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) [29]. Protein concentration was 0.20-0.25 mg/mL in 50 mM ammonium bicarbonate, pH 8.0. Mean residue mass ellipticities were calculated based on 113.26 as the average molecular mass/residue, obtained from the amino acid containing of these proteins, and expressed in terms of  $\Theta$  (degree  $\times$  cm<sup>2</sup>  $\times$  dmol<sup>-1</sup>). CDNN CD spectra deconvolution software (Applied Photophysics, Leatherhead, UK) was used to deconvolute the CD spectra recorded.

#### *Carbohydrate detection and deglycosylation treatment*

Carbohydrate detection on protein after SDS-PAGE transferred to nitrocellulose membranes was performed by using a biotinylated concanavalin A (Con A) lectin solution [30] followed by horseradish peroxidase-labelled streptavidin diluted 1:5000. The staining was developed with 0.05% diaminobenzidine/0.03% of 30% H<sub>2</sub>O<sub>2</sub> in 0.5 M NaCl, 50 mM Tris, pH 7.5 reaction buffer. Enzymatic deglycosylation was performed with PNGase F

endoglycosidase (Boehringer, Mannheim, Germany) as described [30]. nOle e 1 glycosylated and rSal k 4 non-glycosylated protein allergens were used as controls.

### *2D-electrophoretic analysis*

2D-electrophoresis was developed in a PROTEAN IEF Cell (Bio-Rad). Isoelectric focusing was achieved under reducing conditions in the presence of 3 mM tributylphosphine by using 7 cm length, pH 3-10 linear ReadyStrip IPG gels (Bio-Rad). After isoelectrofocusing, proteins were separated by SDS-PAGE in 15% polyacrylamide gels under reducing conditions in the presence of 50 mM dithiothreitol and 3.7% iodoacetamide. 2D-gels were alternatively stained with silver reagent or transferred to nitrocellulose membranes.

### *Immunologic analysis*

Immunodetection on membranes was achieved by using two Ole e 1-specific mAbs (diluted 1:5000), a pAb raised against nOle e 1 (diluted 1:5000), individual sera or a pool of sera from patients sensitized to *S. kali* pollen (diluted 1:10) as described [15]. The IgG binding was alternatively detected with a horseradish peroxidase-labelled goat anti-rabbit IgG diluted 1:3000 (Bio-Rad), or a horseradish peroxidase-labelled goat anti-mouse IgG diluted 1:5000 (Pierce Chemical Co.), and the binding of human IgE was detected with a mouse anti-human IgE antibody diluted 1:5000 (ALK-Abelló) followed by horseradish peroxidase-labelled goat anti-mouse IgG (diluted 1:5000) [9]. For immunoblotting inhibition assays, 0.5 µg of purified protein or 50 µg of pollen extract were incubated with a pool of sera (diluted 1:10) previously incubated with 500 µg of pollen extracts from *S. kali*, *C. album* and *O. europaea*, or 5 µg of purified proteins. The signal was detected using ECL Western blotting reagent (Amersham Biosciences, Barcelona, Spain).

IgE-binding quantification was performed by ELISA in microtiter plates coated with 100 ng/well of protein [31]. Plates were incubated with allergic sera (diluted 1:10). The binding of human IgE was detected as above. IgG-binding by ELISA was detected with monoclonal antibodies against Ole e 1 followed by a horseradish peroxidase-labelled goat anti-mouse IgG (diluted 1:5000) or, alternatively, with a polyclonal antiserum against Ole e 1 followed by a horseradish peroxidase-labelled goat anti-rabbit IgG (diluted 1:3000). The reaction was developed with o-phenylenediamine and the optical density (OD) measured at 492 nm. The OD values under 0.1 were considered negative.



For ELISA inhibition assays the plates coated with 100 ng of purified protein were incubated with a pool of sera (diluted 1:10) previously incubated with different concentrations of purified allergens or *C. album*, *S. kali* and *O. europaea* pollen extracts as inhibitors, and the signal was developed as above.

Serum from a non-atopic individual was used as negative control in the experiments.

## Results

### *Identification of a 19 kDa protein band with IgE-binding reactivity*

Twenty-eight patients allergic to *S. kali* pollen from Murcia and twenty-nine from Zaragoza were recruited to study the allergenic profile of *S. kali* protein extract (fig. 1a). Immunoblots obtained with individual sera showed protein bands from 10 to 70 kDa. A high number of sera recognized a band with a molecular mass of approximately 40 kDa, mostly corresponding to the pectin methylesterase allergen Sal k 1 [9]. In addition, other IgE-reactive proteins with apparent sizes of 13-15, 19 and 25-27 kDa were observed.

A protein band of 19 kDa was recognized by 14 sera (4, 5, 11, 19, 21, 22, 23 in the upper panel and 2, 6, 9, 11, 15, 26, 27 in the bottom panel). The molecular mass fitted well to those of Ole e 1-like protein family. The putative presence of a homologue in *S. kali* pollen was analyzed by immunostaining of the *S. kali* extract under reducing conditions with an antibody raised against Ole e 1 on a 2D-electrophoresis (fig. 1b). A set of IgG-binding spots confirmed this protein as Ole e 1-homologue and showed the polymorphic character of this protein. To determine if this protein has any IgE cross-reactivity to Ole e 1-like proteins and considering the cross-reactivity is restricted to the phylogenetic-related species, inhibition assays were performed with Che a 1, an Amaranthaceae member of this family, and Ole e 1, an Oleaceae allergen using a pool of the above mentioned sera (fig. 1c). The abolishment of 80% of the IgE-binding signal with Che a 1 was observed after quantifying the bands of the blot by densitometry, indicating that this protein share IgE epitopes with its Ole e 1-like counterpart Che a 1.

### *Isolation and molecular characterization of the nSal k 5 allergen*

Three chromatographic steps, one gel permeation step by Sephacryl S-100, one hydrophobicity separation in phenyl-sepharose column and one reverse phase HPLC were carried out to purify the protein at homogeneity. A profile of the last chromatographic step is shown (fig. 2a). MS analysis rendered a broad peak with a maximum at 17628 Da (fig. 2a inset). The protein migrated as a single band of 19 kDa after SDS-PAGE analysis and the glycosylated character was assessed by Con A lectin staining (fig. 2b). This allergen was named Sal k 5 following the criteria of the Allergy Nomenclature Subcommittee of the Immunological Societies.

The IgG-binding ability of Sal k 5 using Ole e 1-specific pAb and two mAbs (OL3 and OL7) antisera, as well as a pool of sera were assayed by immunoblotting (fig. 2c). From the mAbs tested, only OL3 directed against a continuous epitope was able to recognize the protein under reducing conditions. On the other hand the IgG binding from pAb was visible under non-reducing conditions and the signal was more intense when 2-ME was added. When a pool of sera from patients sensitized to *S. kali* pollen was used, a marked decrease of the IgE-binding signal was observed in the presence of 2-ME. Therefore, the disruption of the disulphide bonds seems to affect in a different manner the IgE binding than the IgG reactivity of Sal k 5.

The pattern of spots of the purified protein obtained after 2D-electrophoretic analysis and immunostaining with the pAb (data not shown) was equivalent than that obtained with the *S. kali* extract (fig. 1b). On the other hand, Edman degradation of purified Sal k 5 rendered a single N-terminal sequence of KGN. The peptide mass fingerprinting and MS/MS sequencing analyses obtained by proteomic analysis of purified Sal k 5 led to sequences VYCDTCR, TMMEGATVSIKCR, VDGDHEDDLCKLVLVK and ANALGFLK. The alignment of these sequences with proteins included in the GenBank<sup>TM</sup>/EMBL data base revealed homology with members of the Ole e 1-like protein family and especially to Che a 1.

#### *Immunological properties of nSal k 5 in comparison to Ole e 1-like homologues*

To assess the cross-reactivity degree with Ole e 1-like allergens belonging to phylogenetically related or unrelated families, an immunoblotting inhibition analysis of the IgE binding to purified nSal k 5 was carried out using the same pool of allergic patients' sera and Che a 1 and Ole e 1 allergens, from Amaranthaceae and Oleaceae families, respectively. An almost complete inhibition of the IgE binding was observed with 5 µg of the Amaranthaceae allergens, either Sal k 5 (100%) or Che a 1 (95%). Using 500 µg of total protein of the respective pollen extracts, an inhibition of 80% and 100% was obtained with *C. album* and *S. kali*, respectively (fig. 3a). Lack of inhibition was observed when the olive pollen extract and Ole e 1 were used as inhibitors. These results were further confirmed by ELISA inhibition using the same proteins and extracts as inhibitors (fig. 3a). No significant correlation was observed between the individual IgE-response to Sal k 5 and Ole e 1 (0.0002) with the 57 sensitized patients to *S. kali* pollen. However, Sal k 5 and Che a 1 showed a correlation of 0.8555, assessing the high cross-reactivity between the Ole e 1-like allergens of the Amaranthaceae family (fig. 3b, c).

### *PCR cloning and sequence analysis*

cDNA was synthesized from total RNA from *S. kali* pollen and used as template for amplification of Sal k 5 by PCR using oligonucleotides designed from internal peptide sequences derived from the peptide fingerprinting analysis of the natural allergen. Two overlapping fragments corresponding to the 5' and 3' ends were used to get Sal k 5-nucleotide sequence including the signal peptide in two rounds of PCR (fig. 4a). Finally, a PCR performed with primers designed from the C- and N-terminal ends of the mature protein rendered the whole encoding sequence of the allergen. The open reading frame of this protein contains 176 amino acids including a signal peptide of 25 residues according to the N-terminal sequence obtained by Edman degradation. The theoretical molecular mass was 16448.6 Da and a pI of 5.02 was obtained without the signal peptide. Sal k 5 contains a putative N-glycosylation consensus site at position 43, being this feature associated to the structural relationships among members of the Ole e 1-like family from phylogenetically related species. The sequence identity of Sal k 5 with Che a 1 was 68%, but only 42%, 26% and 32% with Phl p 11, Pla l 1 and Ole e 1, respectively (fig. 4b).

### *cDNA expression and purification of rSal k 5*

The cDNA encoding the mature sequence of Sal k 5 was sub-cloned into the pPICZ $\alpha$ A expression vector. The construction pPICZ $\alpha$ A/Salk5 was used to transform *P. pastoris* KM71 cells to produce the secreted allergen by means of the AOX1 promoter induction with a yield of 40 mg/liter of culture of the extracellular medium. A major band of approximately 21 kDa was visualized by SDS-PAGE. After dialysis of the culture medium, a three-step procedure, by ionic exchange (DEAE-cellulose), size exclusion (Sephadex G-75), and RP-HPLC chromatographies, was performed to purify the recombinant protein (fig. 5a).

### *Validation of rSal k 5 with its natural counterpart nSal k 5*

Structural and immunological comparisons of nSal k 5 and rSal k 5 were carried out to assess the integrity of the recombinant form. To that end, CD analyses of both forms were performed in the far-UV. The ellipticity of nSal k 5 with rSal k 5 was almost equivalent, giving very similar values of secondary structure elements (fig. 5b). MS analysis of rSal k 5 rendered a major peak of 18561.4 Da, and several minor peaks with differences of 160-162.5 Da, which would correspond to different mannose residue content (fig. 5c). rSal k 5 was

treated with PNGase F endoglycosidase to assess its N-glycosylation character. Treated and untreated samples were subjected to SDS-PAGE (fig. 5d). No deglycosylation of the natural protein was achieved, which suggests the presence of fucose monosaccharides in the glycan structure as also occurs with Ole e 1, where fucose-containing oligosaccharide are not removed by PNGase F. However, a partially deglycosylated recombinant form was observed from a mannose-rich glycan.

A comparative analysis of the IgG- and IgE-binding activities of rSal k 5 and nSal k 5 was performed by immunoblotting and ELISA. IgG binding of mAb and pAb to both proteins were similar and therefore no significant differences could be attributed to the polymorphism of the natural protein (data not shown). The eighteen sera, which resulted positive to purified nSal k 5 were tested for IgE binding by ELISA (fig. 6a) and immunoblotting (fig. 6b) to both natural and recombinant proteins, showing a close correlation between both forms ( $r = 0.78$ ). Nine sera recognized better rSal k 5 and nine nSal k 5 by ELISA. IgE binding was also analyzed by means of ELISA inhibition with an equivolumetric pool of 5 sera. nSal k 5 and rSal k 5 were coated in ELISA plates and 5  $\mu$ g of both allergenic variants were used as inhibitors (fig. 6c). The shape of the two curves was similar when rSal k 5 was coated in the wells, whereas rSal k 5 was not able to reach a total inhibition of the IgE-binding to nSal k 5 coated in the wells, indicating the contribution of the specific glycosidic group of nSal k 5 to the IgE binding, or alternatively the absence of any polypeptide epitope in the recombinant form.

## Discussion

The geographic distribution of pollen from different species is strongly influenced by geo-climatic conditions and thus contributes to the sensitization profile of the allergic patients living in a particular area [32]. In the semi desert south-eastern areas from Spain, *Salvadora* is the first cause of seasonal allergy in parallel to olive or grass pollinosis [4,6]. In central Spanish areas, where extensive irrigation is turning grass areas into semi desert ones, a significant increase in *Salvadora* allergy concomitant to a decline in grass allergy is observed [4,33].

Three key points should be addressed to highlight the relevance of *Salvadora* pollinosis: i) their resistance to drought in warm countries, ii) the qualitative and quantitative differences in the allergenic character among the Amaranthaceae species extracts, *S. kali* and *C. album*, although they have been indistinctly included in the diagnosis tests for many years [6], and iii) the overlap of the pollination season of these species with those of other allergenic pollens [34] that play a significant role in the sensitization of patients from these regions.

Amaranthaceae IgE-binding profiles are complex. The immunoblotting of *S. kali* protein extracts with individual sera from patients allergic to Amaranthaceae pollens rendered many IgE-reactive bands, whose identities have not been completely elucidated. Different IgE-binding profiles were detected from individual blots of both populations used in this study. Patients from Zaragoza recognized a higher number and variety of IgE-reactive bands than those from Murcia, whereas the latest ones recognized preferentially bands of 19, 28 and 40 kDa. The band of 40 kDa is Sal k 1, which has been considered a specific marker to the primary sensitization to *S. kali* because it has not been detected in other plants of the Amaranthaceae family such as *C. album* [9]. However, significant inter and intra-species cross-reactivity has been attributed to different allergens already described in this family such as profilins -Che a 2 and Sal k 4-, or polcacinins -Che a 3- [7,8,15,35].

Ole e 1-like protein family contains numerous allergenic members belonging to very different pollens from weeds, grasses and trees. Biological studies pointed out to a relevant function in the pollen grain activity [36]. The knowledge that most of them have been described as allergens allows us suggesting that an intrinsic allergenicity could be associated to either common structural motives or to biochemical capacity. However, the recent characterization of a new member from birch pollen without allergenic properties seems to weaken this theory [24]. In this report, we have identified a new allergen from *S. kali* pollen belonging to the ubiquitous Ole e 1-like family, which can explain the IgE cross-reactivity with other Amaranthaceae members.

Interestingly, the cross-reactivity between Ole e 1-like members has been always associated to those phylogenetically related allergens [17,37-39]. The reason for these IgE-binding differences is based on their amino acid sequence, which is determinant for the interaction between the IgE immunoglobulin and the allergen. Thus, the identities among Oleaceae members -Ole e 1, Lig v 1, Syr v 1, and Fra e 1- are around 85%, and among Amaranthaceae allergens -Che a 1 and Sal k 5- is 68%. However, a high sequence variability exists between Sal k 5 and the Ole e 1-like allergens from phylogenetically non related species -Ole e 1, Pla l 1 or Phl p 11- being 32%, 26%, and 42%, respectively the identity degree. These low values notably reduce the possibility of IgE cross-reactivity among these allergens [40]. In fact, the important amino acid sequence differences observed between Ole e 1 and Sal k 5 could explain the low IgG- and IgE-reactivities between these two allergens and the fact that epitopes hidden in the native structure of the allergen are exposed to the IgG binding only when the unfolding of the molecule is produced. These results were also observed when Ole e 1 and Che a 1 were immunologically compared [17]. Here, we observed similar findings pointing out to the high-cross reactivity between phylogenetically related allergens and an opposite situation for phylogenetically non-related allergens. The IgE binding to Sal k 5 using a pool of sera rendered a complete inhibition when Sal k 5 and *S. kali* extract were used as inhibitors, and a nearly complete inhibition was reached with Che a 1 and *C. album* extract. Neither Ole e 1 nor *O. europaea* extract were able to inhibit the signal.

Finally, a prevalence of more than 30% for natural Sal k 5 purified from pollen was reached in the two populations of sera herein analyzed from patients allergic to *S. kali* indicating the importance of Sal k 5.

The yeast *P. pastoris* has been selected as the expression system to produce rSal k 5. Stability, glycosylation and disulphide bond formation are structural features to consider when choosing the expression system to produce a protein. The success of this selection is herein simplified by the fact that many members of this family had been efficiently produced in this eukaryotic system [17]. Sal k 5 was synthesized and secreted to the extracellular medium of the yeast cells. The recombinant protein displayed a correct folding as determined by spectroscopic analysis and was also glycosylated. However, the molecular mass difference between the natural and the recombinant protein is assumed by a bigger size of the oligosaccharide incorporated in the N-glycosylation consensus site of Sal k 5, which should be composed of a core of N-acetylglucosamine and a group of 10 manose residues for the higher intensity peak and 1 extra manose of 160 Da for each additional peak for rSal k 5 (fig. 5c), in contrast to nSal k 5, where fucose monosaccharides were present in the glycan

structure as also occurs in Ole e 1 glycosylated molecules. However, the recombinant allergen with a putative rich-mannose glycan component added by the yeast is partially deglycosylated by the enzyme.

The glycosylation consensus site in these proteins seems to be coincident in all members of the same botanical family. Thus, a common N-glycosylation consensus site at Asn43 or Asn39 for Sal k 5 and Che a 1, respectively, was observed. However, a different site for the Oleaceae proteins –at Asn110 in Ole e 1, Syr v 1, Fra e 1 and Lig v 1- or Gramineae – at Asn24 in Lol p 11 or Phl p 11- have been identified. Interestingly, the birch member of this family, which neither is glycosylated nor contains a consensus site in its amino acid sequence, is the only Ole e 1-like member described so far which lacks the IgE-binding ability [24]. Other structural feature that defines this family of allergens is their polymorphism, which was also observed for Sal k 5 by 2D-PAGE, although significantly lower than that observed for Ole e 1 with more than 10 different isoforms sequences identified [41].

An essential requisite to include a recombinant allergen in a diagnosis protocol is its accurate validation of structural and immunologic parameters, mainly its IgE-binding capacity, and taking as reference the natural molecule. However, in most cases this comparison is exclusively made using the complete pollen extract where the natural allergen is expressed but in so low amounts that its isolation is a hard task. Herein, the availability of both forms of Sal k 5 allowed us accurately performing these analyses. The spectroscopic data as well as the immunologic inhibition assays confirmed the integrity of the recombinant protein and the presence of most of the B cell epitopes. The lack of a total inhibition of the IgE binding to nSal k 5 using rSal k 5 as inhibitor suggests a possible involvement of the glycan moiety in the IgE reactivity since a different carbohydrate component is incorporated in the N-glycosylation site by the yeast. Other possibility could be the absence of critical residues within the B epitopes in the recombinant isoform.

In summary, according to previous and here presented results, *S. kali* pollen extract seems to be enough for the diagnosis and treatment of most representative Amaranthaceae pollen allergies, since it contains a specific and major allergen (Sal k 1) and is able to inhibit the IgE binding of all the other already known allergens from *C. album* pollen. Moreover, the availability of Sal k 5, together with Sal k 1, and panallergens Sal k 4 and polcalcin as molecular tools to establish a diagnostic algorithm, could allow to stratify patients depending on their sensitization: Sal k 1 as marker of allergy to *S. kali* pollen, Sal k 5 as index to Amaranthaceae sensitization, and finally Sal k 4 and polcalcin as markers of polysensitization. Furthermore, the correlation between the increase of this pollinosis and the



desertization of the soil as well as the relevance of this new member of Ole e 1-like allergen from *S. kali* makes Sal k 5, and the other allergens of *S. kali* pollen suitable candidates to optimize diagnosis protocols.

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## Legend to the Figures

**Fig.1.** Identification of a 19 kDa protein in *S. kali* pollen protein extract as an allergenic molecule belonging to the Ole e 1-like family. (a) Analysis of the IgE-binding from individual allergic patients to *S. kali* pollen extract from two Spanish populations (Zaragoza, n=29 and Murcia, n=28). (b) Silver staining of the protein extract (100 µg of total protein) after 2D-electrophoresis, and immunoblotting with Ole e 1-specific IgG after transference to nitrocellulose membranes. (c) Immunoblotting inhibition assay of the specific binding of a pool of sera to *S. kali* pollen extract using 5 µg of Che a 1 and Ole e 1 allergens as inhibitors. -, no inhibitor. Molecular markers are shown.

**Fig.2.** Purification and characterization of the natural allergen nSal k 5 from *S. kali*. (a) Reverse-phase HPLC was the last step in the isolation of nSal k 5. Horizontal bar represents peak fractions where Sal k 5 eluted. Mass spectrometry of the purified protein is shown in the inset; a.u: arbitrary units. (b) Electrophoretic mobility of nSal k 5 and carbohydrate detection. CBS, Comassie Blue staining and Con A, concanavalin A staining. rSal k 4 non-glycosylated protein was used as negative control. (c) Immunostaining analysis under non-reducing (-) and reducing (+) conditions with 2-ME of the IgG binding by monoclonal OL3 (mAb) and polyclonal (pAb) antibodies raised against Ole e 1, and IgE-binding with a pool of sera and a non-atopic control (N). Molecular markers are shown.

**Fig.3.** IgE cross-reactivity between allergens from the Ole e 1-like family. (a) Top, immunoblotting inhibition assays of the IgE binding to nSal k 5 with purified proteins, or pollen extracts from *S. kali* (Salsola), *C. album* (Chenopod) and *O. europaea* (Olive). Molecular markers are shown. Bottom, ELISA inhibition assays of the IgE binding to nSal k 5 with 0.5 µg and 5 µg of purified allergens, or 50 µg and 500 µg of *S. kali*, *C. album* and *O. europaea* pollen extracts. (b and c) Regression plot of the specific IgE values of the 57 patients' sera measured by ELISA to nSal k 5 in comparison with those of Ole e 1 or Che a 1. Calculated correlation ( $r^2$ ) observed for nSal k 5 vs Ole e 1 and nSal k 5 vs Che a 1 was included.

**Fig.4.** Amino acid and nucleotide sequences of Sal k 5. (a) Nucleotide sequence and deduced amino acid sequence of the cDNA encoding Sal k 5. Peptide sequences showing

homology to peptides obtained by mass spectrometry are highlighted in grey. The amino acid sequence in *italics* corresponds to the signal peptide, the arrow indicates the processing site. (b) Amino acid sequence alignment of Sal k 5 and other members of the Ole e 1-like family: Sal k 5 (GQ427676), Che a 1 (Q8LGR0), Ole e 1 (C53806), Fra e 1 (AY652744), Syr v 1 (S43242), Lig v 1 (O82015), Pla l 1 (AJ313166), Phl p 11 (Q8H6L7). Percentages of identity (I%) and similarity (S%) using Sal k 5 as reference are shown.

**Fig.5.** Molecular comparison of recombinant and natural purified Sal k 5. (a) Coomassie Blue staining (CBS) after SDS-PAGE. (b) Far-UV CD spectra.  $\alpha$ h: alpha helix;  $\beta$ s: beta sheet;  $\beta$ t: beta turn; np: non-periodic conformation. (c) MS of rSal k 5, a.u: arbitrary units. (d) Deglycosylation of both forms compared to nOle e 1 by endoglycosydase F. Molecular markers are shown.

**Fig.6.** Analysis of the IgE binding to natural and recombinant Sal k 5 of 18 sera from allergic patients. (a) Specific IgE-binding values measured by ELISA. (b) Immunoblotting staining. N, a non-atopic serum was used as negative control. (c) IgE ELISA inhibition assays with nSal k 5 coated-wells (left panel) and rSal k 5 coated-wells (right panel), using a pool of sera and the same proteins as inhibitors; insets show the same inhibition experiment of each panel performed by immunoblotting. -, no inhibitor.

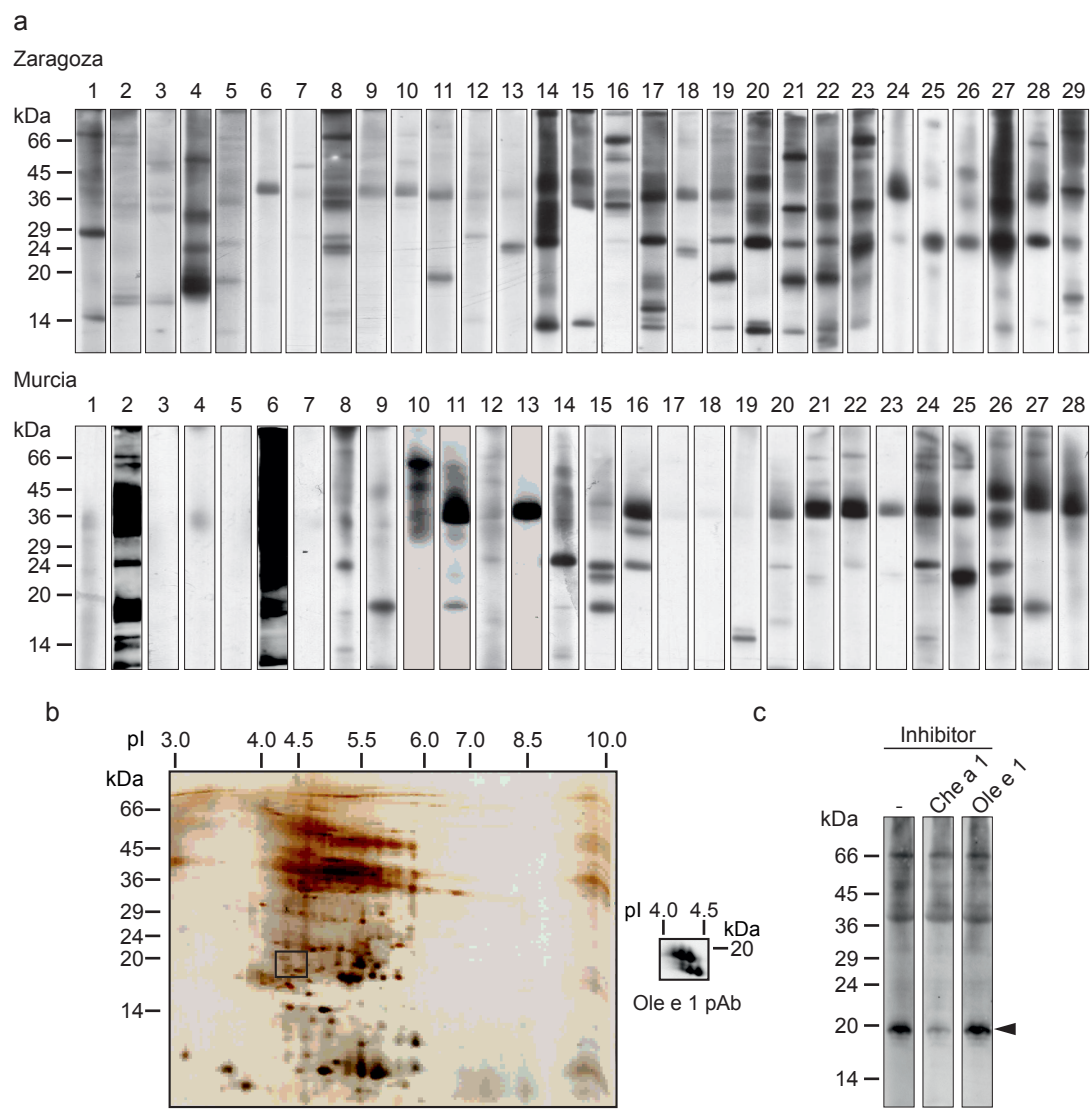


Figure 1

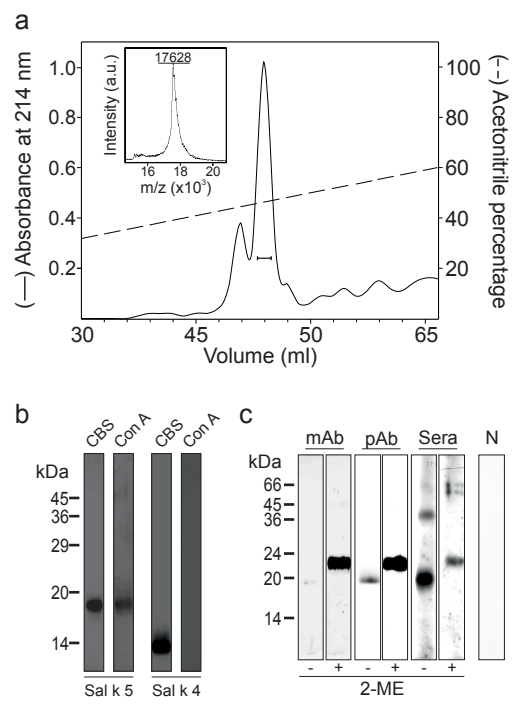
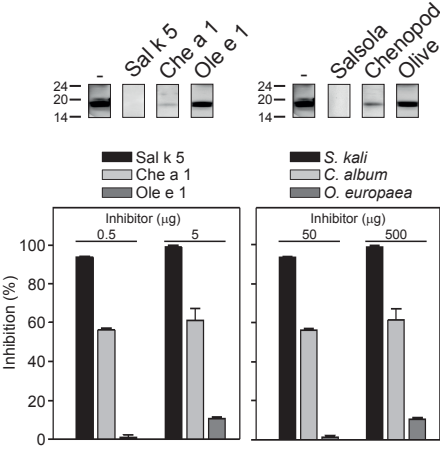


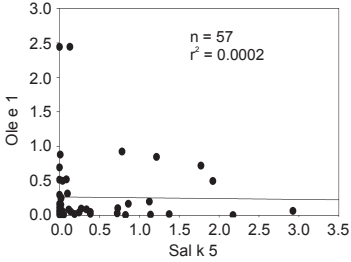
Figure 2



a



b



c

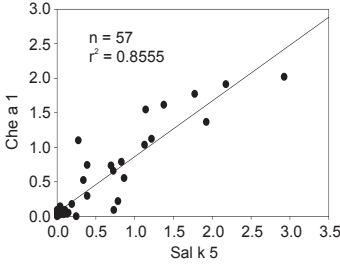


Figure 3

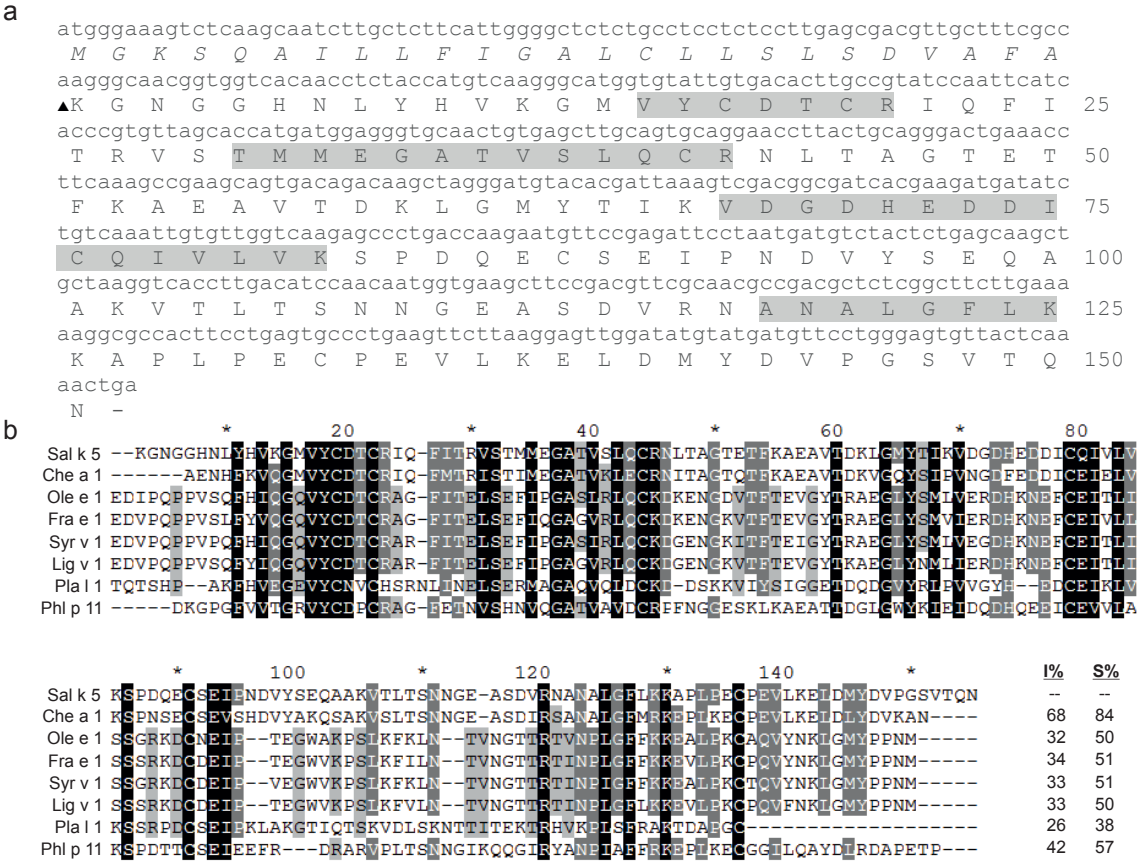


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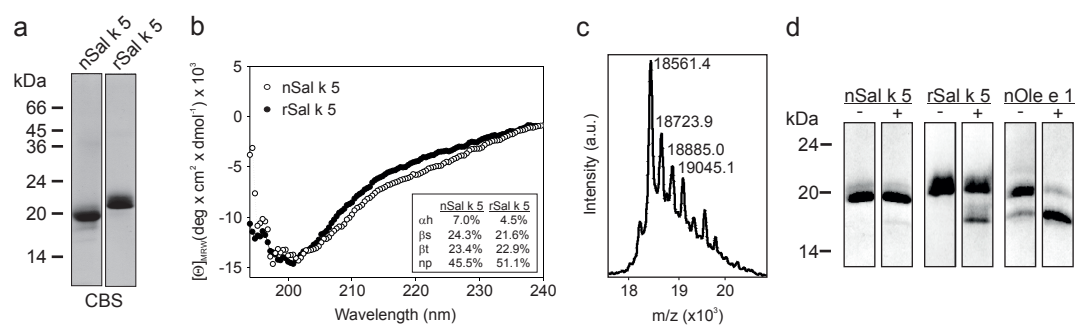


Figure 5

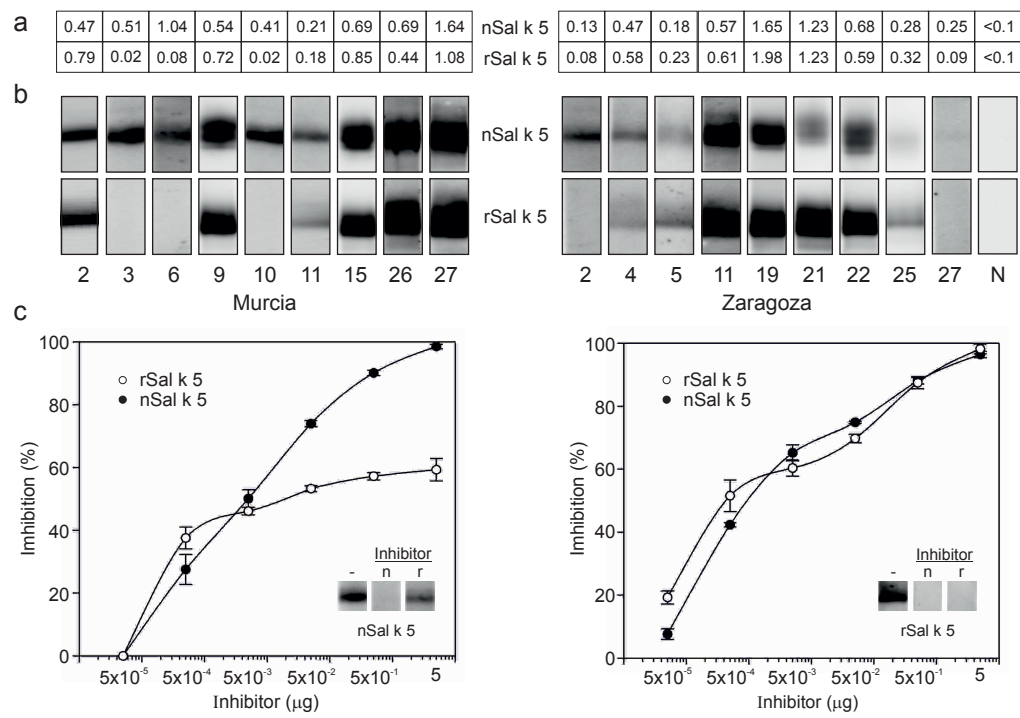


Figure 6